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Regulation of ATRIP protein abundance by RAD9 in the DNA damage repair pathway

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Abstract

Genotoxic stress activates checkpoint signaling pathways that activate the checkpoint kinases ATM and ATR, halt cell cycle progression, and promote DNA repair. A number of proteins act in concert with ATR to phosphorylate Chk1, including RAD17, the RAD9-RAD1-HUS1 complex, ATR/ATRIP and TopBp1. However, how these proteins involved act in concert with one another to propagate and maintain the checkpoint response is not well understood. Here, we reported that upregulation of RAD9 protein increased the quantity of ATRIP, suggesting that RAD9 activation will induce more efficient accumulation of ATRIP in vivo. Furthermore, the DNA damage-induced ATRIP foci formation was faster in the m*Rad9*$^{-/-}$ ES cells. Also, ATRIP interacts specifically with RAD9, but not HUS1 and RAD1. Taken together, we suggested that RAD9 could affect both the ATRIP protein levels and DNA damage-induced ATRIP foci formation. Thus, we propose a role of RAD9 in the ATR-Chk1 pathway that is necessary for successful formation of the damage-sensing complex and DNA damage checkpoint signaling.

Key words: DNA damage signaling, ATRIP, RAD9, Cell cycle checkpoints, Genome stability.

Introduction

Cellular DNA is constantly exposed to DNA damaging caused by environmental factors and intracellular metabolic processes. In response to the genotoxic stress, cell developed cell checkpoint and repair mechanisms, which halt replication, presumably in order to allow time to repair damaged DNA (1-3). The initiation of these checkpoints relies on the efficient recognition of the DNA damage followed by rapid signal transduction by transducer kinases ATR/ATM (RAD3-related kinase/ the ataxia telangiectasia-mutated) (4-6). Where ATM specifically targets double stranded DNA breaks, ATR is involved in a wider array of damage types (7-9). Once activated, ATM and ATR phosphorylate and activate their effector kinases, Chk2 and Chk1, respectively. Chk2 and Chk1 then proceeds to phosphorylate a variety of proteins involved in DNA repair, DNA replication, and cell cycle transitions.

The activation of the ATR pathway by DNA damage and replication stress is a multistep process. The initiation of this process relies on the efficient recognition of the ssDNA by the ssDNA protein binding complex RPA (replication protein A), which facilitates recruitment of ATR to sites of DNA damage through an interaction with the ATR-associated protein ATRIP (10-13). Simultaneously, the checkpoint clamp loader RAD17-RFC complex loads the RAD9-RAD1-HUS1 (9-1-1) complex to the ssDNA independently, followed by binding of topoisomerase binding protein 1 (TopBp1) (14, 15). Thereby 9-1-1 complex contacts with the ATR-ATRIP heterodimer through TopBp1 (16-18). Then, ATR becomes fully active, resulting in the activation of Chk1, thereby allowing Chk1 to phosphorylate a variety of proteins (19-22).

Many DNA damage proteins are recruited to sites of damage into nuclear foci, and become immobilized in these foci due to interactions with either the damaged chromatin or other proteins in the DNA lesion. However, recent studies have indicated that these nuclear foci are not static but hightly dynamic structures (23, 24). Photobleaching experiments revealed an increase in the dynamic behavior of RAD9 within remaining foci in the absence of ATR/ATRIP (20). Indeed, it is reported that DNA damage repair (DDR) foci are highly mobile and coalesce into repair centers in both budding yeast and mammalian cells (25-27). Moreover, recent work has indicated that, under certain conditions, 9-1-1 can be recruited to DNA in a TopBP1-dependent manner (2, 22). If ATRIP recruits TopBP1, as shown in the study by Choi et al. (2, 28), then ATR-ATRIP should also recruit 9-1-1. Obviously, it is unclear how these DNA damage proteins act in concert with one another to propagate and maintain the checkpoint response (23, 29). We need more detail regarding how the ATR-Chk1 pathway operates in living cells. Here, we try to analyze the regulation of ATRIP protein abundance by RAD9, and assess the probable novel role of RAD9 in the ATR-Chk1 pathway.

Materials and Methods

Antibodies

A monoclonal anti-FLAG M2 antibody (F1804), a polyclonal anti-FLAG antibody (F7425), an anti-GAPDH and anti-β-Actin monoclonal antibody were obtained from Sigma-Aldrich (Shanghai, China), while an anti-HA antibody, an anti-MBP antibody and polyclonal anti-ATR (N-19) were obtained from Santa Cruz Technology (Shanghai, China). A polyclonal rabbit anti ATRIP (NBP1-19365) was purchased from Novus Biologicals, and anti-RAD9 monoclonal antibody (611324)

from BD. Anti-TopBP1 (A300-111A) was obtained from Bethyl Laboratories Inc. The mouse polyclonal antibody against HUS1 used was produced within the laboratory. A FITC-conjugated goat anti-rabbit IgG antibody was obtained from Jackson Immuno Research Laboratories (West Grove, PA).

Cell culture

HeLa and HEK 293T cells were cultured in DMEM (Invitrogen, CA) supplemented with 10% fetal bovine serum (Hyclone) and 100U/ml penicillin/streptomycin. HEK 293T cells stably expressing RAD9-FF-ZZ, hHUS1-FF-ZZ and GFP-FF-ZZ, constructed in our laboratory, were cultured in DMEM with 10% fetal bovine serum and 50mg/ml Hygromycin B. The method employed for culturing mouse ES cells has been described elsewhere (30).

Cloning

The pFLAG-CMV2-RAD9, pFLAG-CMV2-hRAD1, pFLAG-CMV2-hHUS1, pFLAG-CMV2-GFP, pcDNA3-6HA-hHUS1 and pGEX-6P-1-RAD9 plasmids have been described previously (31, 32). The full-length human ATRIP sequence was PCR amplified and inserted into both pcDNA3-6HA and pMAL-C_2X. The C terminal double epitope FF-ZZ tag - a tandem construct consisting of two FLAG tags (FF) followed by two protein-A immunoglobulin G (IgG) binding domains (ZZ) from pINX-C-FF-ZZ-A (33) was first cloned into pcDNA3.1(+) to form pcDNA3.1-FF-ZZ. The *Rad9*, *hHus1* and *GFP* sequences were inserted into pcDNA3.1-FF-ZZ.

Establishing protein over-expression stable cell lines

The recombinant vectors (pcDNA3.1-FF-ZZ-RAD9, pcDNA3.1-FF-ZZ-HUS1 and pcDNA3.1-FF-ZZ-GFP) was transfected into 293T by liposome, respectively. The positive transfected cell clones were selected with neomycin. After culture expansion, the stable cell lines were attained. The expressions of RAD9, HUS1 and GFP in the stable cell lines were detected by western blot assay, respectively.

Immunoprecipitation

Transfection of HEK 293T cells was carried out using the Lipofectamine Plus reagent (Life Technologies). The cells were grown to 60-80% confluence in 60mm tissue culture dishes before being transfected with 1.5mg pFLAG-CMV2 DNA and 1.5mg pCDNA3-6HA DNA, following the procedure described by Invitrogen. Immunoprecipitation and Western blotting were performed as described elsewhere (34).

Chromatin fractionation

Chromatin fractionation was performed essentially as described elsewhere (35). A population of ~3×10^6 cells was rinsed in PBS and re-suspended in 200μL 10mM HEPES (pH 7.9), 10mM KCl, 1.5mM $MgCl_2$, 0.34M sucrose, 10% glycerol, 1mM DTT, 10mM NaF, 1mM Na_2VO_3 and a cocktail of protease inhibitors (solution A). Triton X-100 was then added to a final concentration of 0.1%, and the cells held on ice for 5min. Cytoplasmic proteins (S1) were separated from nuclei (P1) via centrifugation (1,300 × *g*, 4min). The P2 preparation was rinsed once in solution A and then lysed in 200μL 3mM EDTA, 0.2mM EGTA, 1mM DTT (solution B). After a 10min incubation on ice, soluble nuclear proteins (S2) were separated from P1 by centrifugation (1,700 × *g*, 4min). Isolated chromatin was rinsed once with solution B and then pelleted (10,000 × *g*, 1min). Finally, the chromatin (P2) was resuspended in 200μL SDS sample buffer and sheared by sonication. To digest chromatin with micrococcal nuclease, the P1 fraction was resuspended in solution A containing 1mM CaCl2 and 50U micrococcal nuclease (Sigma). After a 2min of incubation at 37°C, the nuclei were lysed and fractionated as above.

Immunofluorescence

ES cells were grown on glass coverslips in 35mm dishes. Fixation and permeablization were performed by treating with 4% paraformaldehyde for 15min, followed by 0.5% triton X-100 for 15min. Blocking was achieved by adding 1% BSA at room temperature for 10min, and then the material was challenged with an anti-ATRIP antibody at room temperature for 1h. After extensive rinsing, the secondary FITC-conjugated goat antirabbit IgG antibody was added and left at room temperature for 1h. Images were captured by a Zeiss LSM510 confocal microscope.

Results

The upregulation of RAD9 enhances ATRIP protein abundance in vivo

It is known that RAD9 can regulate levels of other proteins, such as *p21*, DDB2 and ITGB1 (36-38). We examined ATRIP protein levels in RAD9 over-expression stable cell lines, to determine whether or not *in vivo* the abundance of ATRIP was affected by that of RAD9. The over-expression of RAD9 (but not of HUS1, another component of 9-1-1 complex) clearly enhanced the level of ATRIP present (Fig. 1A). Further, to evaluate the regulation of ATRIP by RAD9 expression level, we monitored the difference of endogenous ATRIP expression level between *mRad9*$^{+/+}$ and *mRad9*$^{-/-}$ ES cells. The overall protein expression level of ATRIP in cytoplasmic proteins (S1) and soluble nuclear proteins (S2) were not affected by the RAD9-deficience, but the amounts of ATRIP on chromatin (P1) were slightly reduced in the *mRad9*$^{-/-}$ ES cells (Fig. 2A). The result indicated that RAD9 activation would induce more efficient accumulation of ATRIP *in vivo*.

Over-expression of RAD9 increased ATRIP protein level, how about TopBP-1 and ATR? We also tested if ATR and TopBP1 protein levels were changed in RAD9 over-expression cells. The expression level of TopBP1 was not affected when the abundance of RAD9 was high (Fig. 1B). Surprisingly, the amounts of ATR were slightly reduced in RAD9 over-expression cells (Fig. 1B).

The DNA damage-induced ATRIP foci formation was faster in the mRad9$^{-/-}$ *ES cells*

We next tested whether ATRIP recruitment is influenced in RAD9-deficient cells. A comparison was made between *mRad9*$^{+/+}$ and *mRad9*$^{-/-}$ ES cells with respect to ATRIP recruitment, following exposure to hy-

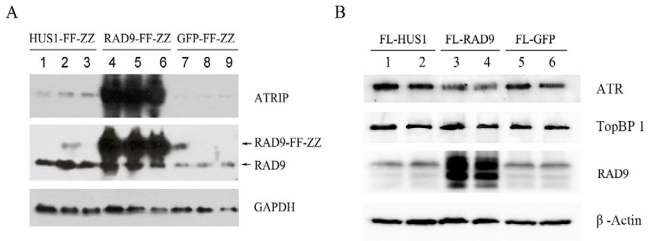

Figure 1. Over-expression of RAD9 regulates protein expression level. A. Over-expression of RAD9 enhances ATRIP protein expression level. pCDNA3.1-RAD9-FF-ZZ, pCDNA3.1-HUS1-FF-ZZ or pCDNA3.1-GFP-FF-ZZ were transfected into HEK 293T cells. Nine random clones were assayed for ATRIP, RAD9 and GAPDH proteins expression. B. Over-expression of RAD9 regulates expression levels of ATR and TopBP1 proteins. pCDNA3.1-FL-RAD9, pCDNA3.1-FL-HUS1 or pCDNA3.1-FL-GFP were transfected into HEK 293T cells, respectively. Extracts of these cells were analyzed by immunoblotting with antibodies to ATR, TopBP1, RAD9 and β-Actin.

droxyurea (HU). ATRIP was still associated with chromatin in the presence of HU, apart from the quantity of ATRIP deposited on the chromatin was slightly reduced when RAD9 was absent (Fig. 2A). In order to ask whether the ATRIP accumulation to the sites of damage can be seen, we observed ATRIP foci formation. ATRIP foci were formed in both *mRad9*$^{+/+}$ ES cells and *mRad9*$^{-/-}$ ES cells exposed to HU for 12h (Fig. 2B).

Furthermore, we studied the kinetics of ATRIP foci formation in HU-induced cells expressing RAD9 or absence of RAD9. We observed faster ATRIP foci

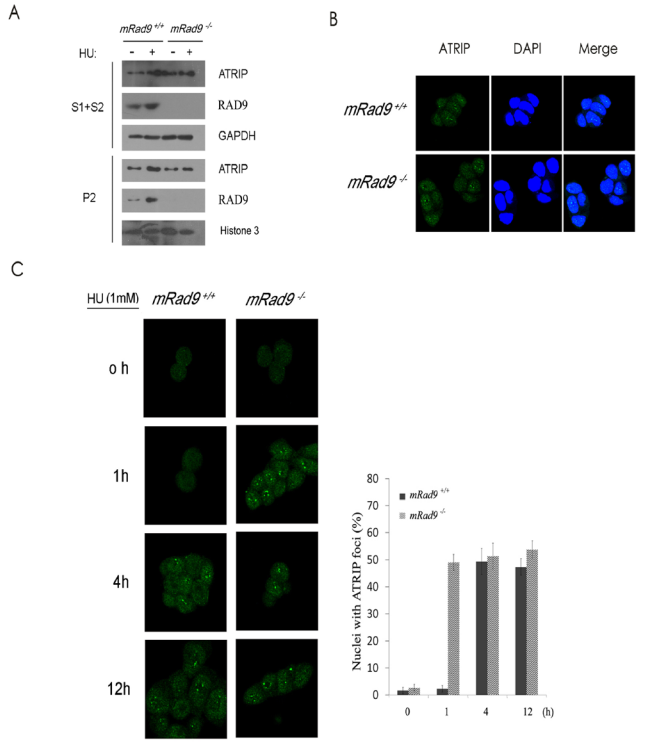

Figure 2. RAD9 deficiency has an effect on ATRIP foci formation at the site of DNA damage. A. *mRad9*$^{+/+}$ and *mRad9*$^{-/-}$ ES cells were untreated or treated with HU, harvested after 12h. The cells were then fractionated, and the resultant fractions were analyzed by immunoblotting with the indicated antibodies. (S1) cytoplasmic proteins; (S2) soluble nuclear proteins; (P2) chromatin-enriched sediment. B. ATRIP localizes to the sites of DNA replication stress exposed to HU for 12h in *mRad9*$^{+/+}$ and *mRad9*$^{-/-}$ ES cells. C. ATRIP localizes to the sites of DNA replication stress exposed to HU for 0h, 1h, 4h and 12h in *mRad9*$^{+/+}$ and *mRad9*$^{-/-}$ ES cells.

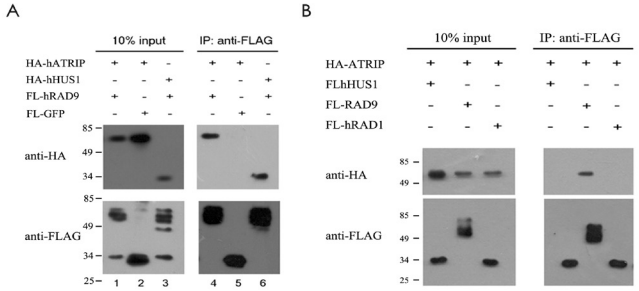

Figure 3. RAD9 interacts with ATRIP. A. Co-immunoprecipitation of FL-RAD9 and HA-ATRIP. pcDNA3-6HA-ATRIP transfected into HEK 293T cells along with: pFLAG-CMV2-RAD9 (lanes 1, 4) or pFLAG-CMV2-GFP (lanes 2, 5: negative control). Lanes 3, 6: pcDNA3-6HA-hHUS1 transfected into HEK 293T cells along with pFLAG-CMV2-RAD9 (positive control). Lysates were immunoprecipitated with anti-FLAG antibody and probed with antibodies recognizing either HA (upper panel) or FLAG (lower panel). B. ATRIP was not associated with either hRAD1 or hHUS1. pCDNA3.0-6HA-ATRIP transfected into HEK 293T cells along with: pFLAG-CMV2-hHUS1 (lanes 1, 4:), pFLAG-CMV2-RAD9 (lanes 2, 5) or pFLAG-CMV2-hRAD1 (lanes 3, 6). Lysates were immunoprecipitated with anti-FLAG antibody and probed with antibodies recognizing either HA (upper panel) or FLAG (lower panel).

formation at sites of damage in *mRad9*$^{-/-}$ ES cells than *mRad9*$^{+/+}$ ES cells exposed to HU (Fig. 2C). We observed ATRIP foci formation in *mRad9*$^{-/-}$ ES cells exposed to 1mM HU for 1h, 4h and 12h, but ATRIP foci formation wasn't observed at earlier time point (1h) in the *mRad9*$^{+/+}$ ES cells, though the difference became smaller at later time points (4h and 12h). Thus, the DNA damage-induced ATRIP foci formation was faster in the *mRad9*$^{-/-}$ ES cells.

ATRIP interacts with RAD9

Above results indicated that ATRIP foci formation was effect by RAD9. We predicted therefore, that ATRIP likely to interact with RAD9 important for the functioning of DNA repair and cell cycle checkpoint control. Co-immunoprecipitation experiments were performed to confirm the association of ATRIP and RAD9 *in vivo.* The assay showed that in cells FLAG-tagged RAD9, the RAD9 protein interacted with ATRIP, but not with the negative control (GFP) (Fig. 3A). A subsequent co-immunoprecipitation assay showed that neither over-expressed FL-tagged hRAD1 nor hHUS1 interacted with HA-tagged ATRIP, even though FL-RAD9 interacted with HA-ATRIP (Fig. 3B). The interpretation of this result was that ATRIP interacts specifically with RAD9, but not HUS1 and RAD1, other two components of 9-1-1 complex.

Discussion

RAD9 regulates multiple DNA damage-inducible cell cycle checkpoints and contribute to different DNA repair pathways, including base excision repair (BER), nucleotide excision repair, mismatch repair and homologous recombination repair (32, 36). In addition to the 9-1-1 complex, RAD9 interacts independently with several DNA repair proteins, including RAD51, MLH1, APE1, TDG, OGG1 and NEIL1 (32, 39, 40). RAD9 can regulate levels of other proteins, such as *p21*, DDB2 and ITGB1 (36-38). Here, we show that ATRIP protein

levels are increase in response to RAD9 over-expression (Fig. 1A), and the amounts of ATRIP on chromatin were slightly reduced in the *mRad9*$^{-/-}$ ES cells (Fig. 2A). . Our data suggests that RAD9 can also regulate ATRIP expression. Why cells need more ATRIP protein when RAD9 protein levels enhance?

In generally, ATR/ATRIP and 9-1-1 complexes are thought to bind to DNA independently, form nuclear foci at sites of DNA damage, and become immobilized in the foci (36, 41). We also observe that ATRIP was still associated with chromatin and form ATRIP foci in in both *mRad9*$^{+/+}$ ES cells and *mRad9*$^{-/-}$ ES cells exposed to HU (Fig 2A, B). Our data indicated that ATRIP recruitment was not influenced by RAD9, supporting the currently accepted dogma that 9-1-1 is not required for ATR/ATRIP to access sites of DNA damage. However, our experiments studying the dynamic behavior of ATRIP foci suggest that although RAD9 does not influence the recruitment of ATRIP to DNA damage sites, it does have an impact on the early kinetics of ATRIP foci formation.

At present, multiple lines of evidence support the idea that nuclear foci are not static but hightly dynamic structures (23, 24). After ATR/ATRIP and 9-1-1 complexes are recruited to DNA lesions, these proteins are highly mobile within DNA damage induced foci. It has been reported that ATRIP associates more transiently with damage chromatin than do the members of the 9-1-1 complex after UV-induced DNA damage (23). In addition to, photobleaching experiments reveal an increase in the dynamic behavior of RAD9 within remaining foci in the absence of ATR/ATRIP (20). Our data show that RAD9 deficient cells would induce more efficient ATRIP foci formtion at the site of DNA damage at the earlier time points (Fig. 2C). Our result demonstrated the effect of RAD9 in ATRIP turnover in established foci. Both of ATRIP and RAD9 were highly mobile at the sites of DNA damaged, turnover of ATRIP within DNA damage induced foci was comparatively slow without RAD9. Thus, it's possible that ATRIP appears to be to stabilize the RAD9 at the sites of DNA damage, resulting in an increase in the mobility of ATRIP in the presence of RAD9 at the initial DNA-damage-induced proteins recruitment stage.

Then, how does ATRIP promote RAD9 stability after DNA damage? We provided evidence that RAD9 interacts specifically with ATRIP (Fig. 3). The prevailing view is that TopBP1 links the independently recruited 9-1-1 and ATRIP/ATR complexes, leading to ATR-mediated Chk1 phosphorylation (21, 41, 42). We propose that ATRIP could bind RAD9 dynamically, and the specifical interaction of RAD9/ATRIP contributes to stabilize the RAD9 at the sites of DNA damage (Fig. 4). But the detailed mechanism remains to be elucidated. Such as, how about the interaction of RAD9 and ATRIP with HU treatment, is the interaction stronger? And how about the interaction of ATRIP with HUS1 and RAD1 when treatment with HU? The effect of HU-induction was remarkable, the overall protein expression level of ATRIP and RAD9 in cytoplasmic proteins (S1) and soluble nuclear proteins (S2) were enhanced in HU-induced cells, and the chromatin association of ATRIP and RAD9 (P1) were also obviously elevated in the HU-induced *mRad9*$^{+/+}$ cells (Fig. 2A). Combined with the fact

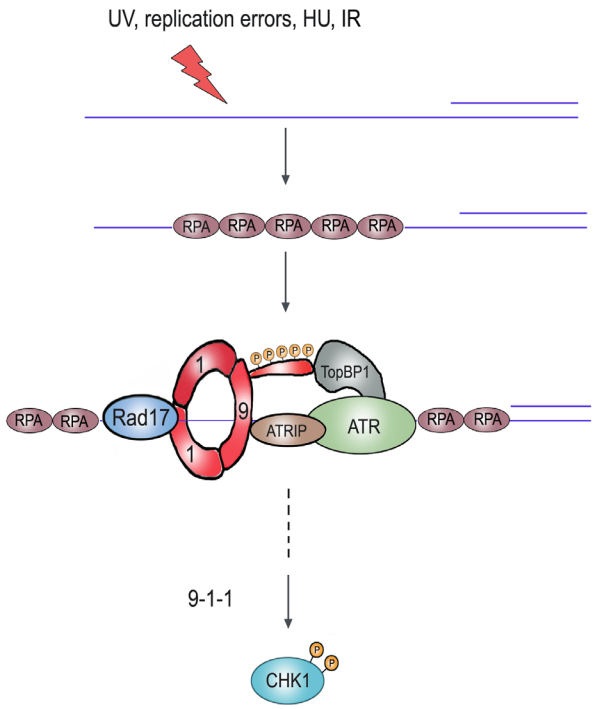

Figure 4. RAD9 interacts with ATRIP for stabilizing the ATRIP/ATR complex at the site of DNA damage. RPA binds to ssDNA, and recruits ATR-ATRIP by interacting with ATRIP. Rad17 complex recruits the 9-1-1 clamp complex to the sites of damage. RAD9 did interact with ATRIP, the ATRIP/RAD9 interaction served to stabilize the association of the ATR-ATRIP complex with chromatin. TopBP1 interacts with ATRIP/ATR and phosphorylated RAD9. Those proteins loaded to the damaged DNA, leading to phosphorylation of the downstream checkpoint kinase-1 (CHK1) and other ATR effectors.

that ATRIP interacts specifically with RAD9, but not HUS1 and RAD1 (Fig. 3B), we could inferred that the interaction of ATRIP and RAD9 with HU treatment was stronger, but not with HUS1 and RAD1.

Overall, we suggested that the upregulation of RAD9 enhance the ATRIP protein levels. ATRIP and RAD9 can be independently recruited to damaged DNA. Once recruited, a specifical interaction of RAD9/ATRIP occurs and affects the foci formation at the sites of DNA damage.

Acknowledgements

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